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Characterization of anti-listerial bacteriocin produced by lactic acid bacteria isolated from traditional fermented foods from Cambodia

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Abstract

Listeria monocytogenes is a foodborne pathogen associated with fermented products. The aim of this study was to characterize anti-listerial bacteriocin produced by lactic acid bacteria (LAB) isolated from Cambodian fermented foods. Four LAB isolates of confirmed bacteriocin production were isolated from fermented fish and shrimp (pha ak trey, pha ak kampus). These isolates demonstrated antimicrobial activity against *Listeria monocytogenes* NCTC 11994 and were identified as *Pediococcus pentosaceus* by 16S rRNA analysis. Based on these results, one isolate was selected (*P. pentosaceus* CFF4) and its bacteriocin was characterized. Complete inactivation or significant reduction in bacteriocinogenic activity was observed after treatment with proteases and α -amylase, suggesting that the bacteriocin besides the proteinaceous nature has the presence of a glycosidic moiety that may be required for its activity. The molecular weight of the bacteriocin was approximately 3.5 kDa, as determined by tricine-SDS-PAGE. The bacteriocin activity was unaltered for pH values ranging from 2 and 10, and temperatures between 4 and 60°C. *Pediococcus pentosaceus* CFF4 could be considered for use as a starter culture producing anti-listerial agents in fermented foods. Further studies are still needed concerning the biopreservative properties of the purified bacteriocin and the organoleptic characteristics of the foods.

Introduction

Despite the long history of fermented foods in Southeast Asia, there is less scientific research on their quality and safety characteristics. Though some studies have been conducted in some countries, it seems the characteristics of indigenous fermented foods have not been widely disseminated. Many of the foods remain artisanally produced by small-scale backyard producers, in contrast to the situation in Japan or in China (Owens, 2015). Similarly, Cambodian fermented foods have been produced traditionally (Chuon *et al*., 2013). Their processing technologies have not been well researched and improved.

Fermented shrimp is known as pha ak kampus in Khmer (Cambodian language). Fresh shrimp is cleaned and mixed with salt. The fermentation takes place in a closed jar under sunlight for five to seven days. Ground roasted rice powder and galangals are added into the jar and the fermentation process continues for two or three days more until it gives sour aroma. The final fermented product has an acidic flavor and is consumed raw right after fermentation; it can be added with little pieces of papaya, sugar and ginger for enhancing taste and aroma. Its shelf life is less than one week, stored at room temperature; it may be longer at lower temperature (4°C) (SreyNin, 2015). Fermented fish or pha ak trey has a similar taste to pha ak kampus and the production process is very similar. Fish is cleaned; the scales and head are removed and then mixed with salt. Fermentation takes at least one week in a closed jar. After mixing with ground roasted rice powder and the fermentation continues until it releases a sour aroma.

Prahok is known as the representative fermented fish product of Cambodia. It has a unique taste and strong flavor being used as seasoning or condiment in Cambodian cuisine (Norng *et al*., 2011; Chuon *et al*., 2013). There are two traditional types of Prahok: bony prahok and boneless prahok produced by small or medium size fish. For the boneless process, removing of bone is required after removing the head and scales. The fish is soaked in water until swollen for about 24 h at room temperature. Then the fish is sun dried for 1 h to remove the water from fish. After

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that, it is mixed with a saline solution and kept in a close jar under the sun for one month. This product can be kept for six months to one year at room temperature. Fermented fish sausage or nem trey is a typical appetizer in Cambodia. Fish meat is mixed with salt, sugar, garlic, steamed rice, pepper, spices and well ground until the fish meat becomes sticky. Then it is placed into plastic wrap and rolled to acquire a beef or pork sausage shape and allowed to ferment for 24 to 48 h. It can be consumed immediately after fermentation or grilled.

Beef sausage or kwa ko is made from ground beef and mixed with some ingredients such as sugar to enhance flavour, steamed rice to accelerate the fermentation process by the action of microorganisms present in cooked rice, garlic, galangal, roasted rice powder and rice wine. After well mixing, it is necessary to fill into pork intestine and keep one day at room temperature. After this it is sun dried for two to three days until it is well dried. After the fermentation the beef sausage has an acidic flavour and a special aroma. It must be well-cooked preferably by grilling or frying.

Pork sausage or sach krok chrok is made from ground pork and mixed with sugar, salt and ground star anise powder. The fermentation takes place in a jar containing white rice wine for two to three days. Then it is packed into pork intestine and sun dried for two or three days more to make sure they are very well dried. Pork sausage is consumed well-cooked as an appetizer.

Fresh cucumber or chrourk tror sok is fermented in a closed container with saline solution. The fermentation takes about two or three days when the cucumber changes colour and gives the acidic flavour. Spices and garlic can be added also to obtain the special aromas of garlic and a spicy taste. Fermented cabbage or chrourk sapei, the cabbage is cleaned (a particular cabbage grown in Cambodia, green leaf) then kept for one or two days under the sun until it becomes dry. It is then placed into a closed container with 5% saline solution and fermented from five days to one week until it produces the desired acidic flavour. Then it is chopped and mixed with garlic, spices, salt and sugar and consumed as a salad.

Fermented rice noodles or nom banh jok, is consumed usually as a substitute for rice in various types of soup. Non-sterilized rice grains are soaked and fermented overnight. Then it is ground and the water removed using mechanical pressure and kept for 12 h until the water is removed. After that, it is cooked and vigorously shaken until the rice becomes sticky and processed into noodles.

The production of these Cambodian fermented foods is performed through the knowledge which passes from person to person and from Cambodian older generations. Therefore, all procedures previously described were obtained by interviewing people that manufacture these products. Fermented foods are part of the typical diet in Cambodia. Certain fermented products can be consumed raw after fermentation. The habit of eating these foods raw may lead to health risk problems if the raw materials are contaminated or if they are contaminated after processing through handling or storage.

Foodborne illnesses resulting from consumption of foods contaminated with *L. monocytogenes* have become the greatest public health concern in the world (Mor-Mur and Yuste, 2010). In particular, outbreaks related to ready-to-eat foods including certain fermented foods due to cross contamination through handling, storage and consumption without prior cooking, have become recognised as a health risk. Hence, using starter cultures or preservatives could reduce the risk related to foodborne illness caused by pathogenic bacteria.

In addition, consumer demands for foods free of chemical additives are also increasing. Therefore, bacteriocins of LAB which are considered as natural biopreservatives and LAB considered as GRAS (Generally Recognized as Safe) microorganisms by the American Food and Drug Administration, should be an alternative to chemical additives ensuring safer food for consumers.

Nisin is currently the only bacteriocin widely used as a food preservative. Many bacteriocins have been characterized biochemically and genetically, and though there is a basic understanding of their structure-function, biosynthesis, and mode of action, many aspects of these compounds are still unknown (Cleveland *et al.*, 2001).

Consequently, this is one of the few studies concerning investigation on bacteriocin producing LAB isolated from Cambodian fermented foods, which should be developed further for improving the quality and safety of fermented foods to control foodborne pathogenic bacteria. This study aimed to characterize the anti-listerial bacteriocins produced by lactic acid bacteria isolated from traditional fermented foods from Cambodia.

Material and Methods

Origin and sampling of fermented products

Nine different Cambodian fermented products were selected and purchased from different vendors in Doeum Ko market in Phnom Penh, Cambodia. A sample from each product (approximately 150 g

each) was packed into a plastic bag and covered by aluminium foil. Samples were transported at room temperature and frozen at -20°C until being analyzed.

Nine products were categorized into four categories: Fermented shrimp and fish products (including pha ak kampus, pha ak trey, prahok, nem trey); Meat products (including kwa ko and sach krok chrok); Fermented vegetables (including chrourk tror sos and chrourk sapei); Khmer fermented fresh rice noodle (nom banh jok).

Screening and isolation of lactic acid bacteria

Screening of LAB was conducted at three different temperatures: room temperature, 30°C and 37°C. One gram of each sample was added to 9 ml of de Man, Rogosa and Sharpe (MRS) broth (Lab M, Bury, UK). Tubes were incubated under microaerophilic conditions for 24 h at room temperature, 30°C and 37°C. Appropriate decimal dilutions were prepared in sterile Ringer's solution (Lab M) and plated onto MRS agar. Plates were incubated at room temperature, 30°C and 37°C under microaerophilic conditions between 48 to 120 h until the colonies developed. Colonies were randomly selected based on their morphology. The selected colonies were purified by repeated streaking onto MRS agar. All isolated colonies were tested for Gram staining, catalase and oxidase production. Gram positive, catalase and oxidase negative colonies were selected and stored at -20°C in MRS broth containing 30% (v/v) glycerol (Panreac, Barcelona, Spain) for further experiments.

Screening anti-listerial activity of lactic acid bacteria isolates

Listeria monocytogenes NCTC 11994 was used as target strain for screening the inhibitory effects of LAB isolates. *Pediococcus acidilactici* HA-6111-2 (bacteriocinogenic strain with antilisterial activity) previously isolated from Alheira (Albano *et al*., 2007; Albano *et al.*, 2009) was used as the control indicator.

The LAB isolates stored at -20 °C and *P. acidilactici* HA-6111-2 were cultured in plates containing MRS agar and *L. monocytogenes* in Trypticase Soy Agar with Yeast Extract (0.6% w/v) (TSA-YE; Lab M). Then, the cultures were grown in 9 ml of MRS broth for LAB isolates and in TSB-YE for *L. monocytogenes* at 37°C for 24 h.

Ten microliters of LAB cultures were spotted on TSA-YE spread with *L. monocytogenes* and incubated overnight at 30°C. Inhibitory activity was recorded as positive if a halo zone was observed around the spot. For those strains demonstrating antimicrobial activity, initial characterization of the antimicrobial activity was performed according to Tomé *et al.* (2006).

Those LAB cultures showing inhibitory activity were centrifuged (Rotina 35R, Hettich, Germany) at 8877 x g for 15 min at 4°C. In order to eliminate the effects of low pH value and hydrogen peroxide on inhibitory activity, cell free supernatant sterilized by membrane filtration (0.22 µm, Orange Scientific, GyroDisc Syringe Filter, Belgium) was neutralized (pH=6.5) with NaOH (1M) and treated with beef liver catalase (Sigma, Steinheim, Germany; 0.1 mg/ml , sterile) for 1 h at 37°C. To determine the proteinaceous nature of the detected antibacterial substances, neutralized cell-free supernatant treated with catalase was treated with trypsin (Sigma, 0.1 mg/ml, sterile) at 37°C for 1 h (Sigma). Cell free supernatant, neutralized cell free supernatant treated with catalase and neutralized cell free supernatant treated with catalase and trypsin were spotted against *L. monocytogenes*, as described above.

Genotypic characterization of the bacteriocin -producing lactic acid bacteria strain DNA extraction from lactic acid bacteria isolates

The total cellular DNA from LAB isolates was extracted using a DNA extraction kit (GRS Genomic DNA kit-Bacteria-GRiSP Research Solutions, Porto, Portugal).

Polymerase chain reaction amplification

16S rRNA genes of the extracted DNA were amplified by Polymerase Chain Reaction (PCR) in a Eppendorf Mastercycler® personal Thermal Cycler System in a final volume of 50 µL containing 1×PCR (Taq buffer +$(NH_4)_2SO_4$ (10x), 25 mM $MgCl_2$, 1 mM of dNTPs, 100 µM of each primer and 1.25 units of Taq DNA polymerase). Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-3') were used.

Amplification conditions were: primary DNA denaturation step at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.30 min at 72°C, with an extension of the amplified product at 72°C for 10 min. The amplified products were separated by electrophoresis in 1% (w/v) agarose gels in 1× TAE buffer (4.84 g Tris-base, 1.09 g glacial acetic, 0.29 g ethylenediaminetetra acetic acid, 1 l distilled water) at 80 V for 18 min. A 100-bp DNA ladder (Bio-Rad Laboratories, Richmond, CA) was used as a molecular weight marker (Abrams *et al.*, 2011). Gels were stained in TAE buffer containing SYBR Green.

PCR products were purified using the GRS PCR & Gel Band Purification Kit (GRiSP), according to

the supplier's instructions, and the DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). The 16S rRNA sequences were edited manually, using the software Bio Edit7.1.3 (IbisBiosciences, Carlsbad, CA, USA; *http://www.mbio.ncsu.edu/bioedit/bioedit.html*). The nucleotide sequences of 16S rRNA gene were compared with reference sequences available in the EzTaxon data base *(http://www.ezbiocloud.net/eztaxon)*.

Characterization of bacteriocin produced by Pediococcus pentosaceus *CFF4*

Based on the bacteriocinogenic activity and genetic similarity, one isolate of LAB (*P. pentosaceus* CFF4) was selected for further studies.

Antimicrobial activity spectrum of Pediococcus pentosaceus *CFF4*

The isolate with confirmed anti-listerial activity was further tested for its antimicrobial activity against other Gram positive bacteria (*Bacillus subtilis* ESBCC 01; *Enterococcus faecalis* ATCC 29212; *Staphylococcus aureus* ESBCC 81) and Gram negative bacteria (*Acinetobacter lwoffii* ESBCC T7BT5; *Acinetobacter junii* UGCC 889; *Acinetobacter pittii* ESBCC T1BP1; *Acinetobacter baumannii* ESBCC 05; *Klebsiella* spp. ESBCC 01; *Escherichia coli* ATCC 25922; *Proteus vulgaris* ESBCC 01; *Salmonella* Typhimurium ESBCC 01; *Pseudomonas* spp. ESBCC 01; *Cronobacter sakazakii* ATCC 51329).

The cell free supernatant was spotted on TSA-YE spread with the target pathogenic strains previously grown at 37°C for 24 h in TSB-YE. The cell free supernatant that showed inhibitory halo was neutralized, treated with catalase and trypsin to check if the inhibition was caused by the activity of the bacteriocin.

The antimicrobial activity of LAB isolate was quantified according to the method described by van Reenen *et al.* (1998). Briefly, a doubling dilution series was made of the cell-free culture supernatant. An aliquot of 10 ml of each dilution was spotted onto TSA-YE plate seeded with actively growing cells of the target organism grown at 37°C for 24 h. Plates were incubated at 30°C. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (van Reenen *et al.,* 1998).

Effect of enzymes, temperature and pH on bacteriocin activity

This study was performed according to the method described by Albano *et al.* (2007). Lactic acid bacteria isolate was grown in MRS broth overnight at 37°C. The cells were harvested (8877 x g, 15 min, 4°C) and the cell-free supernatant adjusted to pH 6.5 with 1 M NaOH. One milliliter of cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml and 0.1 mg/ml of proteinase K (Sigma), pronase (Sigma), papain (Sigma), pepsin (Sigma), α-amylase (Sigma), catalase, and trypsin respectively (Albano *et al*., 2007). Antimicrobial activity was monitored by using the agar-spot test method (van Reenen *et al.*, 1998).

The effect of pH on the activity of bacteriocins was tested by adjusting cell-free supernatants from pH 2.0 to 12.0 (at increments of two pH units) with sterile 1 M NaOH or 1 M HCl. After 1 h of incubation at room temperature (25°C), the samples were re-adjusted to pH 6.5 with sterile 1 M NaOH or 1M HCl and tested for antimicrobial activity by using the agar- spot test method.

The effect of temperature on bacteriocin activity was tested by incubating cell-free supernatants, adjusted to pH 6.5 at 4, 25, 30, 37, 45, 60, 80, and 100°C for 60 and 120 min, and at 121°C for 20 min. The agar-spot test method was used in all tests. Enterococcus faecalis ATCC 29212 and *L. monocytogenes* NCTC 11994 were used as target pathogenic bacteria.

Partial purification and molecular size of bacteriocin

Pediococcus pentosaceus CFF4 was cultured in 300 ml MRS broth at 37 °C overnight. Cells were harvested (8877 x g, 10 min, 4°C) and ammonium sulphate gradually added to the supernatant to 40%, 60%, 80% saturation respectively. After 4 h of slow stirring at 4 °C, the proteins were harvested (8877 x g, 10 min, 4°C). Precipitated proteins in the pellet and floating on the surface were collected and solubilized in 25 mM ammonium acetate buffer (pH 6.5). All samples were stored at -20 °C.

In a separate experiment, the purified bacteriocin was separated by Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (tricine-SDS PAGE) (Schägger and von Jagow, 1987). A low molecular weight marker with sizes ranging from 1.060 to 26.600 kDa (Sigma) was used. The gels were fixed and one half was stained with Coomassie Brilliant Blue R250 (Saarchem, Krugersdorp, South Africa). The positions of the active bacteriocins were determined by overlaying the other half of the gel (not stained and extensively pre washed with sterile distilled water) with cells of *L. monocytogenes* embedded in TSB-YE agar (1% agar w/v).

Results and Discussion

Screening and isolating lactic acid bacteria

One hundred isolates presumptively identified as LAB (Gram positive, catalase negative, oxidase negative) were recovered from nine products: forty isolates were recovered from fermented fish and shrimp products, followed by twenty-eight isolates from fermented meat products, twenty-three isolates from fermented vegetables and nine isolates from Khmer fermented noodle.

Screening of anti-listeria activity of lactic acid bacteria isolates

For the first culture screening method, among one hundred LAB isolates, there were seven LAB isolates recovered from fermented fish (pha ak trey) and fermented shrimp (pha ak kampus) producing a translucent halo around the spot, inhibiting the growth of *L. monocytogenes*. All the seven LAB isolates were recovered from samples incubated at 37°C. These inhibitory effects may result from the competition by LAB, lactic acid production with consequent pH decrease, hydrogen peroxide or bacteriocin produced by LAB (O'Bryan *et al.*, 2015). According to the screening method used, only four LAB isolates produced proteinaceous compounds that demonstrated antimicrobial activity (activity was lost after the treatment with trypsin). These results suggested the anti-listerial activity was caused by a bacteriocin. The other three isolates showed antimicrobial activity by competition (activity was lost after centrifugation) (Table 1).

Some studies found anti-listeria bacteriocin producing LAB strains, *E. faecium* CN-25 in traditionally Thai fermented fish roe, som-kai-pla (Sonsa-Ard *et al*., 2015). While, bacteriocin, amysin, produced by *Bacillus amyloliquefaciens* was isolated from Thai shrimp paste (Kaewklom *et al*., 2013). No strains demonstrating anti-listerial activity were found in fermented fish sausage, fermented meat, vegetable and rice fermented noodle. In contrast, some researchers isolated anti-listeria bacteriocin producing LAB from these fermented foods (Noonpakdee *et al*., 2003; Castro *et al.*, 2011; Wang *et al*., 2014; Fontana *et al*., 2015; Gao *et al*., 2015; Sonsa-Ard *et al*., 2015).

Identification of bacteriocin-producing lactic acid bacteria

The four isolates recovered from fermented fish and shrimp that produced bacteriocin were identified as *P. pentosaceus* by 16S rDNA sequencing. Similarly, the isolates from small fish fermented with boiled rice in Myanmar were identified as *Lactobacillus plantarum*-group, *Lb. farciminis, Lb. futsaii, Lb. reuteri, Weissella paramesenteroides*, and *P. pentosaceus* (Moe *et al*., 2015). In contrast, the LAB isolated from fermented fish roe were identified as *E. faecium* CN-25 which shows activity against *L. monocytogenes* TISTR 1327 (Sonsa-Ard *et al*., 2015). Some studies found *P. pentosaceus* in Idly batter, a traditional fermented food in South India (Vidhyasagar and Jeevaratnam, 2013); in Alheira, a traditional fermented and lightly smoked sausage of Portugal (Abrams *et al.*, 2011); in Sichuan Pickle, a traditional pickle of China (Huang *et al*., 2009); in Kimchi, a traditional fermented vegetable of Korea (Jang *et al.*, 2014). As all bacteriocin-producing LAB isolated in our study were identified as *P. pentosaceus,* only one isolate (*P. pentosaceus* CFF4) was selected for the bacteriocin characterization.

Table 1. Anti-listerial activity of lactic acid bacteria isolated from fermented shrimp (pha ak kom pes) and fermented fish (pha ak trey)

Product	Producer strain	CS	NF	NCF	NFCT
Fermented shrimp (*pha ak kampus*)	CFS1	+	+	+	-
	CFS20	+	+	+	-
	CFS 19	-	-	-	-
Fermented fish (*pha ak trey*)	CFF4	+	+	+	-
	CFF5	+	+	+	-
	CFF 9.1	-	-	-	-
	CFF 14.4	-	-	-	-
Control: *Pediococcus acidilactici* HA-6111-2		+	+	+	-

*+ inhibition zone; - no inhibition zone; CS, cell free supernatant; NF, cell free supernatant adjusted to pH 6.5; NCF, cell free supernatant adjusted to pH 6.5 and treated with catalase; NFCT, cell free supernatant adjusted to pH 6.5 and treated with catalase and trypsin.

Antimicrobial activity spectrum of Pediococcus pentosaceus *CFF4*

The antimicrobial activity of *P. pentosaceus* CFF4 against other Gram positive and Gram negative bacteria was further investigated. The results indicated that this isolate shows activity against only Gram positive bacteria, including *B. subtilis* ESBCC 01 and *E. faecalis* ATCC 29212. There was no significant activity against Gram negative bacteria. After cell-free supernatant was treated with trypsin, the activity was lost for *E. faecalis* ATCC 29212; therefore this inhibition was caused by the bacteriocin production. The bacteriocin activity was 800 AU/ml against *L. monocytogenes*, but only 200 AU/ml for *E. faecalis* ATCC 29212.

Table 2. Effect of enzymes treatment on bacteriocin activity

Enzyme	*Listeria monocytogenes*	*Enterococcus faecalis*
Proteinase K 1.0 and 0.1mg/ml	-	-
Pepsin 1.0 and 0.1mg/ml	-	-
Trypsin 1.0 and 0.1mg/ml	-	-
Pronase 1.0 and 0.1mg/ml	-	-
Papain 1.0 and 0.1mg/ml	-	-
Catalase 1.0 and 0.1mg/ml	+	+
α-amylase 1.0 and 0.1mg/ml	-	-

* - No inhibition zone; + inhibition zone

Table 3. Effect of temperature on bacteriocin activity

Temperature *faecalis*	*Listeria monocytogenes*	*Enterococcus*
4°C, 1 h and 2 h	+++	+
25°C, 1 h and 2 h	+++	+
30°C, 1 h and 2 h	+++	+
37°C, 1 h and 2 h	+++	+
45°C, 1 h and 2 h	+++	+
60°C, 1 h and 2 h	+++	+
80°C, 1 h	++	-
80°C, 2 h	-	-
100°C, 1 h and 2 h	-	-
121°C, 20 min	-	-
Control (room temperature)	+++	+

* -, No inhibition zone; +, diameter of the inhibition zone < 1cm; ++, diameter of inhibition zone = 1cm; +++, diameter of the inhibition zone > 1 cm

Effect of enzymes, temperature and pH on bacteriocin activity

The antimicrobial activity of bacteriocin was completely inactivated after incubating for 2 h in the presence of concentrations of 1.0 mg/ml and 0.1 mg/ml of the enzymes trypsin, pronase, proteinase K, pepsin and papain (Table 2). This is an expected result due to the protein nature of bacteriocins (Huang *et al.*, 2009; Wang *et al.*, 2014). The bacteriocin also completely lost the activity after incubation with α-amylase., suggesting that the bacteriocin has a protein nature and the presence of a glycosidic moiety that might be required for its activity (Sivakumar *et al.*, 2010).

It was observed that cell-free supernatants of *P. pentosaceus* CFF4 cultures that were incubated at different temperatures ranging from 4°C to 60°C for 1 h and 2 h exhibited >1cm diameter of inhibition zones for *L. monocytogenes* NCTC 11994 and <1 cm diameter of inhibition zone for *E. faecalis* ATCC 29212, similar to the control samples (maintained at room temperature) (Table 3). These results indicated that the antibacterial substance was stable at temperatures ranging from 4°C to 60°C. Incubation at 80°C for 1 h decreased activity against *L. monocytogenes* NCTC 11994 (diameter of the inhibition zone = 1 cm); activity was completely lost after 2 h at 80ºC. However, after incubation at 80°C for 1 h and 2 h the bacteriocin lost activity against *E. faecalis* ATCC 29212.

In addition, incubation at 100°C for 1 h and 2 h and 121°C for 20 min completely inactivated the activity against L. monocytogenes NCTC 11994 and *E. faecalis* ATCC 29212. In contrast, many other studies have shown that heat treatment at 80°C, 100°C and 121°C did not affect the antibacterial activity of *P. pentosaceus* against *L. monocytogenes* (Abrams *et al*., 2011; Jang *et al*. 2014).

The effect of pH on the antibacterial activity was tested by changing the pH of the cell-free supernatants over a range of 2 to 12. Results showed that the antibacterial activity of the culture supernatants did not exhibit a significant difference after treatment at pH values ranging from 2 to 10 values. At pH 12 the bacteriocin completely lost activity against *L. monocytogenes* NCTC 11994 and *E. faecalis* ATCC 29212 (Table 4).

Table 4. Effect of pH on bacteriocin activity

pH *faecalis*	*Listeria monocytogenes*	*Enterococcus*
2	+++	+
4	+++	+
6	+++	+
8	+++	+
10	+++	+
12	-	-
Control (pH 6.5)	+++	+

* -, No inhibition zone; +, diameter of the inhibition zone < 1cm; ++, diameter of inhibition zone = 1cm; +++, diameter of the inhibition zone > 1 cm

Similar to our results, several studies have reported that antibacterial substances are stable over a wide range of pH values, from 2 to 10 (Kingcha *et al.*, 2012; Biscola *et al*., 2013; Wang *et al*., 2014). However, at a pH value of 12, the antibacterial activity decreased (Noordiana *et al*., 2013). Considering food applications, the tolerance of this bacteriocin over the wide ranges of pH values would allow it to be used in low-pH food and even alkaline food (Gao *et al*., 2015; Woraprayote *et al*., 2015).

In addition to their sensitivity to heat, this bacteriocin could be used in low heat food processing or no prior cook fermented foods like fermented shrimp or fermented vegetables as a natural anti-listeria agent to improve the quality and safety of traditional foods (Jang *et al.,* 2014). However, further

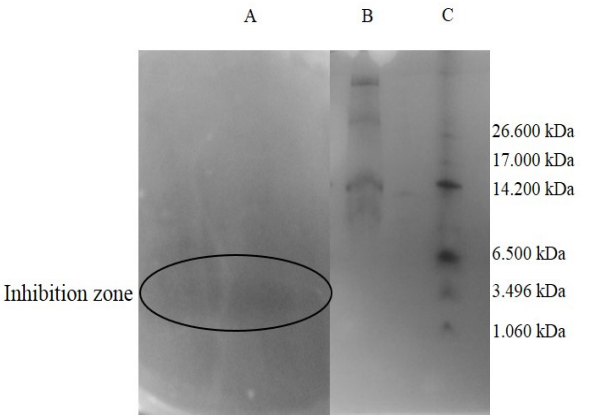

Figure 1. Tricine-SDS-Gel of bacteriocin and overlay; (A) represents the overlay with *Listeria monocytogenes*, (B) peptide band stained with Coomassie Blue R250 and (C) molecular weight marker

researches on the characteristics of bacteriocins as a biopreservative and the organoleptic features of the fermented products are necessary.

Partial purification and molecular size of bacteriocin produced by Pediococcus pentosaceus *CFF4*

The molecular size of the bacteriocin produced by *P. pentosaceus* CFF4 is approximately 3.5 kDa, as determined by tricine-SDS PAGE (Figure 1). This is within the range of most bacteriocins reported for the genus *Pediococcus* (Papagianni and Anastasiadou, 2009).

Conclusions

Pediococcus pentosaceus CFF4, isolated from fermented Cambodian traditional foods demonstrated antimicrobial activity against *L. monocytogenes* NCTC 1199 and *E. faecalis* ATCC 29212 by the production of a bacteriocin. This bacteriocin maintained antimicrobial activity at low and high temperatures (< 60°C) and over a wide pH range (pH 2-10); it was also sensitive to proteases and α-amylase. Our results provide useful information on the potential applications of LAB isolates from fermented fish and shrimp for the development of starter cultures or natural anti-listeria agents. However, further research should be conducted in the future especially on the characterization of purified bacteriocin and its application as a biopreservatives.

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